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A selective culture medium for screening linezolid-resistant gram-positive bacteria

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A B S T R A C T

The SuperLinezolid medium was developed for screening resistance to linezolid (LZD) in Gram-positive bacteria (*Staphylococcus* spp., *Enterococcus* spp.). It was evaluated using LZD-susceptible (n = 20) and LZD-resistant (n = 17) Gram-positive isolates. The sensitivity was found to be 82% at 24 h (3 out of 17 isolates being missed), and reached 100% at 48 h. At 48 h, a single LZD-susceptible isolate grew (specificity 95%). By testing stools spiked with LZD-resistant Gram-positive strains, an excellent performance of the medium was observed, with a lowest detection limit ranging from 10^1 to 10^2 CFU/ml. Overall, this medium is accurate for detection of LZD-resistant Gram-positive isolates after 24 h of culture.

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Linezolid
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Oxazolidinone antibiotics such as linezolid (LZD) are increasingly used as a consequence of an increased rate of multidrug-resistant Gram-positive pathogens (Leach et al., 2011). Considering the wide diffusion of methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates worldwide (Purrello et al., 2016), as well as the wide diffusion of vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis* (Zahedi Bialvaei et al., 2017), there is a significant need to rely on the use of LZD to treat infections caused by those multidrug-resistant isolates (Cattoir and Giard, 2014). Therefore, it not surprising that occurrence of LZD-resistant isolates is now increasingly reported, including in *S. aureus, S. epidermidis, E. faecium*, and *E. faecalis* (Bi et al., 2017; Bourgeois-Nicolaos et al., 2014; Gu et al., 2013). Consequently, accurate and rapid identification of LZD-resistant isolates is needed.

The main mechanism of resistance to LZD in Gram-positive bacteria corresponds to a specific mutation (G2576 T) in the 23S rRNA gene, preventing the binding of the drug to its target, i.e. the ribosome (Sadowy et al., 2018). Some other mutations have been reported in the 23S rRNA gene, leading to modifications of the L3, L4, and L22 ribosomal proteins, but they are much less frequent (Bi et al., 2017; Gu et al., 2013; Pfaller et al., 2017a, 2017b).

In addition, acquired transferable LZD resistance genes have been reported, namely the *cfr*, *cfr*(B), *cfr*(C), *optrA*, and *poxtA* genes (Sadowy, 2018). The *cfr* gene encodes a methylase modifying the C-8 position of A2503 residue in the 23S rRNA methylases (Kehrenberg et al., 2005; Schwarz et al., 2000), and confers resistance not only to LZD but also to phenicols, lincosamides, pleuromutilins, and streptogramins (so called $PhLOPS_A$ resistance phenotype), but noticeably spares the novel oxazolidinone tedizolid (Long et al., 2006). This gene has been identified in *S. aureus*, enterococci, *Streptococcus suis*, and *Bacillus* spp., but also in several Gram-negative bacteria such as *Escherichia coli* and *Proteus vulgaris*. The *cfr*(B) gene sharing 72% nucleotide identity with *cfr* was found in *S. aureus*, also conferring a $PhLOPS_A$ resistance phenotype (Marín et al., 2015). The *cfr*(C) gene encoding a protein sharing ca. 55% amino acid identity with Cfr(A) and Cfr(B) was found in *Campylobacter* spp. (Tang et al., 2017).

In addition to those Cfr-like 23S rRNA methylases, another acquired resistance trait to LZD has been reported, being OptrA (Wang et al., 2015). It belongs to the ABC-F family of ATP-binding proteins, and has been characterized as a ribosomal protection protein. The corresponding gene was first identified in *E. faecalis*, and later in *Staphylococcus sciuri* and *Streptococcus gallotycus* (Sharkey et al., 2016).

Finally, another ribosomal protection protein, PoxtA, was recently identified from a human methicillin-resistant *S. aureus* (MRSA) clinical isolate isolate, and further identified in *E. faecalis* and *E. faecium* of

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Table 1
Preparation of the SuperLinezolid medium.

Compounds	Stock solution	Quantity or volume to add[a]	Final concentration
BHI agar powder	-	14.8 g	3.7%
Distilled water	-	400 ml	
Linezolid	2 mg/ml	300 µl	1.5 µg/ml
Aztreonam	10 mg/ml	80 µl	2 µg/ml
Colistin sulfate	15 mg/ml in water in glass tubes	400 µl	15 µg/ml
Amphotericin B	20 mg/ml in D(+)-glucose 10%	100 µl	5 µg/ml

[a] The volume of 400 ml of SuperLinezolid medium was for twenty plates.

animal origin (Antonelli et al., 2018). That resistance mechanism confers reduced susceptibility not only to oxazolidinone, but also to tetracyclines and phenicols.

Taking in account the potential clinical threat represented by a diffusion of LZD-resistant strains, our aim was to develop a selective culture medium for screening of LZD-resistant bacteria both among human and animal isolates.

1. Material and methods

1.1. Preparation of the SuperLinezolid medium

The necessity to prevent contamination by Gram negatives and fungi was taken in account for the development of this medium. Based on our own experience of developing screening media (Nordmann et al., 2012, 2016), the optimal medium retained was based on the Brain Heart Infusion (BHI) medium (ref 3,564,014; Bio-Rad, Cressier, Switzerland). This medium was rich enough to enhance the growth of the Gram-positive isolates we tested.

To determine the optimal concentrations of each compound of the SuperLinezolid medium, a series of different preliminary tests was performed, using six linezolid-resistant and two linezolid-susceptible isolates, including *Staphylococci* and *Enterococci* strains. Using an inoculum with an optical density of 0.5 Mac Farland (inoculum of ~10^8 CFU/ml), a 1000-fold dilution of the strains to be tested was made in normal saline solution and a 100-µl volume was plated onto the SuperLinezolid medium. To quantify the viable bacteria in each dilution, BHI agar medium was inoculated concomitantly with 100 µl of suspension and incubated overnight at 37°C. A range of concentrations varying from 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 mg/L was tested, and the 1.5 mg/L concentration was retained since it allowed to obtain the optimal sensitivity and specificity values. Colistin sulfate (tablets, MAST DIAGNOSTICS, Merseyside, UK) was added in the medium at a final concentration of 15 µg/ml to prevent the growth of colistin- susceptible Gram-negative isolates. In addition, considering that growth of colistin-resistant Gram-negative strains might be a source of contamination (particularly with species being intrinsically resistant or heteroresistant to colistin such as *Proteus* spp., *Serratia* spp., *Hafnia* spp., or *Enterobacter cloacae*) (Poirel et al., 2017), aztreonam was added at a concentration of 2 µg/ml. While addition of aztreonam contributed to inhibit growth of Gram-negative bacteria (unless they produced broad-spectrum ß-lactamases), it did not modify the growth of the Gram-positive bacteria tested, including LZD-susceptible and -resistant isolates. Amphotericin B (Bristol-Myers-Squibb, Rueil-Malmaison, France) was added as an antifungi molecule at a final concentration of 5 µg/ml. Cultures were incubated at 37°C during 18 h. When no growth was observed after 24 h, the incubation period was extended to 48 h to definitely assess that no growth actually occurred.

The instructions for the preparation of SuperLinezolid medium are indicated in Table 1. The stock solutions may be kept at −20°C. For preparing the SuperLinezolid medium, the diluted powder of BHI was autoclaved at 121°C for 15 min. After cooling this medium for one hour at 56°C, the antibiotic stock solutions were added (Table 1). Once poured, the plates were stored at 4°C and protected from direct light exposure. The SuperLinezolid plates were kept one month and no major effect on its performances was noticed over time (data not shown). (See Fig. 1.)

A total of thirty-seven isolates of various Gram-positive species (*Enteroccocus faecium*, *Enterococcus faecalis*, *Enterococcus casseliflavus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus capitis*) recovered in France were tested to evaluate the performance of the SuperLinezolid medium. Seventeen of those isolates were resistant to LZD, and twenty isolates were susceptible. In addition, a total of eleven Gram-negative isolates were tested, including Enterobacteriaceae (n = 9), *Pseudomonas aeruginosa* (n = 1), and *Acinetobacter baumannii* (n = 1) isolates. All those isolates were clonally-unrelated (data not shown).

The lowest limit of detection with the SuperLinezolid medium was determined for all the tested strains. Using an inoculum with an optical density of 0.5 McFarland standard (inoculum of ~10^8 CFU/ml), serial 10-fold dilutions of the isolates were made in normal saline, and 100-µl portions were plated onto the SuperLinezolid medium. To quantify the viable

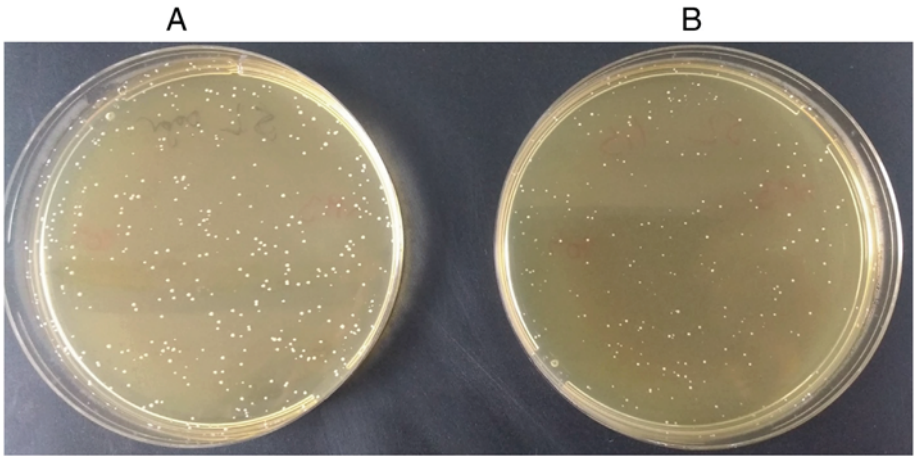

Fig. 1. LZD-resistant *S. epidermidis* (A), and *E. faecium* (B) growing onto the SuperLinezolid medium.

Table 2
Lowest limits of detection of the SuperLinezolid medium for linezolid-resistant isolates.

Strains	MIC of linezolid (mg/L)	Growth on the SuperLinezolid medium At 24 h	At 48 h
E. faecium CNR-15-307	32	+	+
S. aureus 2015S421	8	+	+
S. aureus ST2015–1386	32	+	+
S. capitis ST 2015–2014	>256	+	+
S. epidermidis ST2014–0603	8	+	+
S. epidermidis A	>256	+	+
S. epidermidis 2	>256	+	+
S. epidermidis 22	>256	+	+
S. epidermidis ST 2015–1734	>256	+	+
S. epidermidis ST 2015–1739	>256	+	+
S. epidermidis LESC	256	+	+
S. epidermidis HM-1	16	−	+
S. epidermidis HM-2	16	−	+
S. epidermidis HM-3	256	+	+
S. epidermidis HM-4	32	+	+
S. epidermidis HM-5	256	+	+
S. epidermidis HM-6	8	−	+
E. faecalis ATCC 29212	2	−	−
E. faecalis N95	1	−	−
E. faecalis N89	1	−	−
E. faecalis 2953	2	−	−
E. faecalis 2094	2	−	−
E. faecalis 2146	2	−	−
E. faecalis 2147	2	−	−
E. casseliflavus 2149	2	−	−
S. aureus ATCC 29213	2	−	−
S. aureus C1013	1	−	−
S. aureus C1014	1	−	−
S. aureus 2954	2	−	−
S. aureus 2092	2	−	−
S. aureus 2973	2	−	+
S. aureus 3108	2	−	−
S. aureus 2959	2	−	−
S. aureus 2732	1	−	−
S. epidermidis N30	1	−	−
S. epidermidis N79	1	−	−
S. epidermidis 2145	2	−	−

bacteria in each dilution, BHI agar was inoculated concomitantly with 100 μl of suspension and was incubated overnight at 37°C. The number of viable colonies was counted after 24 h of culture at 37°C. The sensitivity and specificity cut-off values were set at 1×10^3 CFU/ml i.e., a limit value of 1×10^3 CFU/ml and above was considered as « not efficiently detected » (Schwarz et al., 2000).

Spiked stools were also tested using this selective culture medium, done in triplicate. Spiked fecal samples were made by adding 100 μl of each strain dilution to 900 μl of fecal suspension that was obtained by suspending 5 g of freshly pooled feces from three healthy volunteers in 50 ml of distilled water, as done previously (Nordmann et al., 2016). A non-spiked fecal suspension was used as negative control. The lowest detection limit was determined by plating 100 μl of each dilution on the screening medium. The sensitivity and specificity were determined using the same cut-off value set at $\geq 10^3$ CFU/mL (Nordmann et al., 2016).

MICs of LZD were determined using the broth microdilution method in Mueller-Hinton broth, as recommended by the CLSI (Clinical and Laboratory Standards Institute, 2018). For each strain, an inoculum corresponding to 5×10^5 CFU/ml was distributed in the 96-well tray (Sarstedt, Nümbrecht, Germany). The evaluation of the selective medium was performed in triplicate.

According to the CLSI and EUCAST breakpoints (www.eucast.org/clinical_breakpoints/), *Staphylococcus* spp. and *Enterococcus* spp. isolates with MIC values of LZD ≤ 4 mg/L are categorized as susceptible, whereas those with MIC values ≥8 mg/L are categorized as resistant.

2. Results

Overall, all LZD-resistant Gram-positive isolates tested grew on the SuperLinezolid medium after 24 h, except three LZD-resistant *S. epidermidis* isolates (HM-1, HM-2, and HM-6) that did not grow after 24 h of incubation (even at a concentration of 10^3 CFU/ml), but grew after 48 h (Table 2). As expected, no growth was observed for the 11 Gram-negative isolates tested.

The sensitivity was found to be 82% at 24 h (3 out of 17 isolates being missed), and reached 100% at 48 h. At 48 h, a single LZD-susceptible isolate grew (specificity 95%).

For all the LZD-susceptible isolates, the lowest limit of detection was found above the cut-off value of 10^3 CFU/ml, being in fact $\geq 1 \times 10^6$ CFU/ml (Table 2). By contrast, all the LZD-resistant isolates grew on the SuperLinezolid medium in 24 h and the lowest limit of detection was below the cut-off value. All LZD-susceptible Gram-positive did not growth after 24 h of incubation when the inoculum was up to 10^3 CFU/ml; small colonies were obtained only for a single isolate (*S. aureus* 2973) after 48 h of incubation when using this same inoculum. Therefore, a specificity of 100% was observed after a 24-h culture. Finally, no Gram-negative isolate grew after either 24 h or 48 h of incubation.

The spiked stools containing LZD-resistant strains grew with a lowest detection limit ranging from 10^1 to 10^2 CFU/ml (Table 3), thus nicely corresponding to the required sensitivity. In addition, no false-positive isolate was recovered, thus showing an excellent specificity.

3. Discussion

We developed here a selective medium allowing screening and detection of Gram-positive bacteria exhibiting resistance to LZD. Considering that LZD-resistant isolates are currently emerging in different parts of the world, this selective medium may be used to perform prospective screening, and epidemiological surveys. Some surveys had used media supplemented with LZD for selection of LZD-resistant isolates but neither systematic evaluation nor development of such media had been performed (Bourgeois-Nicolaos et al., 2014; Lode et al., 2001).

The SuperLinezolid medium may detect all clinically-relevant bacterial species exhibiting resistance to LZD, regardless of the resistance

Table 3
Lowest limits of detection of the SuperLinezolid medium for a series of linezolid-resistant isolates in spiked stools.*

Strains	Species	Genotype	MIC of linezolid (μg/ml)	Lowest limit of detection
2015S421	*S. aureus*	Cfr+	8	10^1
CNR	*E. faecium*	CfrC + OptrA+	32	10^1
ST2015–2014	*S. capitis*	Mutations RNA T/2319/C; G/2576/T	>256	10^2
ST2014–0603	*S. epidermidis*	Mutations RNA T/2504/A; C/2534/T	8	10^1
LESC	*S. epidermidis*	ND (cfr-; optrA-)	256	10^1
HM-2	*S. epidermidis*	ND (cfr-; optrA-)	16	10^{1*}
HM-3	*S. epidermidis*	Cfr+	256	10^1
HM-6	*S. epidermidis*	Cfr+	8	10^2

* Only after 48 h growth.

mechanism, and regardless of their resistance level to LZD. Growth of most isolates showing MICs of LZD ranging from 8 to >256 μg/ml on this selective medium was confirmed. These resistant isolates were either *S. aureus, S. epidermidis, E. faecium, or S. capitis.* Failure to detect any LZD-resistant isolate tested was observed in three cases after a 24-h culture, corresponding to *S. epidermidis* isolates having an MIC at 8 or 16 μg/ml, therefore close to the breakpoint. The sensitivity was improved and all isolates detected at 48 h; however, the specificity at 48 h was lower, with 6 out of 20 LZD-susceptible isolates being recovered. It seems therefore that a 24-h growth is recommended. An extended evaluation of this selective medium is needed to guarantee high specificity and high sensitivity of the selection. We may admit that our study was performed with a limited number of isolates (due to limited isolates available in our collection), nevertheless we believe that the performances of the SuperLinezolid medium as obtained here do have a significant value.

This medium offers the possibility either to assess the LZD resistance feature by selecting such isolates after re-striking of mixed cultures, but most of all to select for those LZD-resistant isolates from stools in a context of direct screening.

Therefore, the use of this selective medium may contribute to rapidly identify carriers of LZD-resistant isolates, and consequently to rapidly implement infection control measures in order to limit their spread in hospital settings.

Finally, this medium may be also useful for screening food-producing animal reservoirs. Indeed, resistance to LZD in Enterococci has been increasingly identified particularly in swine, poultry and cattle, mainly in China (Fang et al., 2018). An animal reservoir of LZD-resistant strains has been demonstrated for a series of bacterial species, including *E. faecalis* and *E. faecium* (pigs and chicken), but also for *Enterococcus gallinarum*, *Enterococcus casseliflavus, Enterococcus thailandicus*, and *Enterococcus suis* (pigs) (Sadowy, 2018). Prospective surveys may therefore be conducted by veterinary networks in order to evaluate the rate of LZD-resistant Gram positives in animal husbandries, and identify possible reservoirs.

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Conflict of interest

None to declare.

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